SABiosciences™

User Manual

BIOMOL GmbH
Waidmannstr. 35
22769 Hamburg
info@biomol.de
www.biomol.de
Phone: +49-40-8532600 or 0800-2466651 (D)
Fax: +49-40-85326022 or 0800-2466652 (D)

APX™ System

Adjustable Protein Expression

Plasmids and Lentiviral Particles

See Purchaser Notification for limited use license and warranty information (page 3).

Part #1050A Version 1.1 10/2/2009

APX System

Adjustable Protein Expression Plasmids and Lentiviral Particles

User Manual

For Catalog Numbers:
DA#-###A
DB#-###A
DN#-###A
DO#-###A

Ordering and Technical Service Contact Information:

BIOMOL GmbH
Waidmannstr. 35
22769 Hamburg
info@biomol.de
www.biomol.de
Phone: +49-40-8532600 or 0800-2466651 (D)
Fax: +49-40-85326022 or 0800-2466652 (D)

You may place orders by fax, e-mail or from our website. Each order should include the following information:

- Your contact information (name, phone, email address)
- Product name, catalog number and quantity
- Purchase order number or credit card information (MasterCard)
- Shipping address
- Billing address

For more information, visit us at **www.biomol.de**

SABiosciences Corporation
6951 Executive Way, Suite 100
Frederick, MD 21703
USA

CONTENTS

I. Background and Introduction

APX System

The APX System is the most advanced technology for adjusting expression levels of exogenous proteins. The APX System provides two levels of flexibility for the scientist. First, expression constructs are available as either constitutively expressed proteins or as Destabilization Domain (DD)-containing proteins that have an adjustable expression. The stability of the fusion protein is regulated in a dose-dependent fashion by the presence of a small membrane-permeable molecule, Centry. Finally, expression constructs are available in either a ready-to-transfect plasmid or as ready-to-transduce lentiviral particles.

This expression system relies on a FKBP-derived Destabilization Domain (DD, 107 residues) that is engineered to be unstable and thus degrade in the absence of its ligand. Genetic fusion of this Destabilization Domain to a protein of interest (POI) confers instability to the entire fusion, resulting in degradation. Binding of a ligand (named Centry) for the Destabilization Domain protects the fusion from degradation, which allows the POI to accumulate in the cell and exert its normal biological function. The ligand does not elicit any detectable off-target effects when administered to cultured cells or animals including humans. Therefore, this system couples the specificity of a genetic fusion with responsiveness a small-molecule ligand can provide.

Benefits:

- Precise control of protein expression level with Centry Reagent
- Ready-to-transduce lentiviral particles or ready-to-transfect DNA enable the use with virtually any mammalian cell
- Flexibility: constitutive protein expression or adjustable expression vectors available
- ORFs have been sequenced and validated

Application – iPSC Generation

The discovery that mouse fibroblasts can be reprogrammed and generate induced pluripotent stem cells (iPSC) with qualities remarkably similar to embryonic stem cells has created a valuable new source of pluripotent cells for drug discovery, cell therapy, and basic research.

SABiosciences has developed expression vectors for six iPSC related proteins (Nanog, Oct4, Sox2, c-Myc, Klf4 and Lin28) to create a resource for producing induced pluripotent stem cells. Ectopic expression of these factors has been shown to create pluripotent cells which resemble embryonic stem cells. The expression vectors for the six iPSC-related proteins are available as either ready-to-transfect plasmids or ready-to-transduce lentiviral particles. In addition, all six iPSC-related proteins are available as either constitutively expressed ORFs or as adjustable versions (APX system).

How to Regulate Protein Expression Directly with the APX System

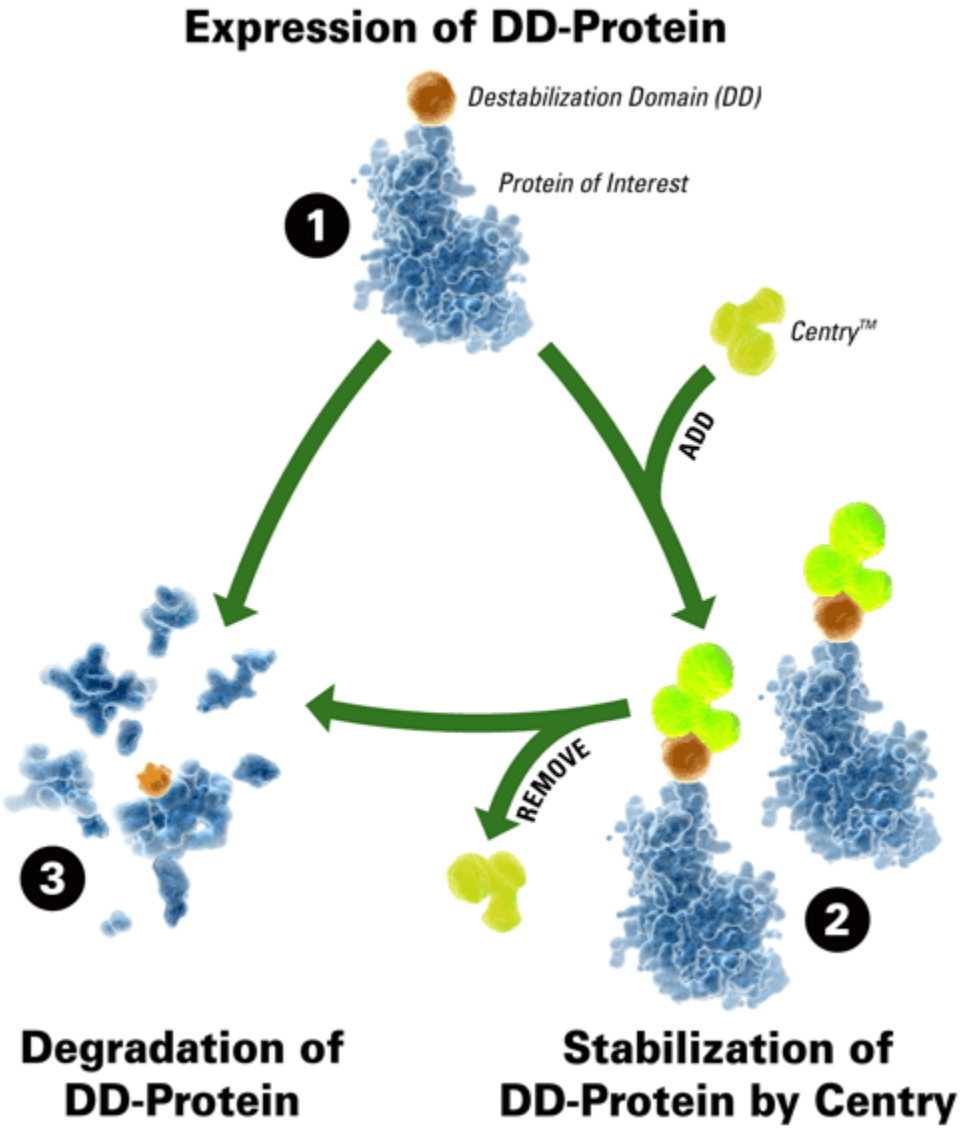


Figure 1. Fusion of a Destabilization Domain (DD) to a protein of interest (POI) confers instability to the entire fusion. Addition of a ligand for DD rescues the fusion protein from proteasomal degradation.

II. Materials Provided:

1. DYKDDDDK*-tagged ORF expression plasmid:

Component/Description	Amount	Concentration	Volume
Ready-to-transfect expression vector	10 μg	500 ng/μl	20 μl

2. DD-tagged ORF adjustable expression plasmid:

Component/Description	Amount	Concentration	Volume
Ready-to-transfect expression vector	10 μg	500 ng/μl	20 μl

3. Lentiviral particles expressing DYKDDDDK*-tagged ORF:

Component/Description	Concentration	Volume
Ready-to-transduce Lentiviral particles	≥ 0.8×10^7 TU/ml	250 μl

4. Lentiviral particles expressing DD-tagged ORF:

Component/Description	Concentration	Volume
Ready-to-transduce Lentiviral particles	≥ 0.8×10^7 TU/ml	250 μl

Storage Conditions:
Ready-to-transfect expression vectors should be stored at -20°C and ready-to-transduce lentiviral expression vectors should be stored at -80°C upon receipt.

Description
Available APX vectors express human or mouse Nanog, Oct4, Sox2, c-Myc, Klf4 or Lin28 under the control of cytomegalovirus (CMV) promoter. For the map of parent vectors, see Appendix.

* The DYKDDDDK epitope tag is also referred to as the FLAG® epitope tag. FLAG is a registered trademark of Sigma-Aldrich Co.

III. Additional Materials Required:

- **Mammalian cell line cultured in the appropriate growth medium**
- **Cell culture medium and standard cell culture supplies**
- **Tissue culture plates**
- **SureFECT™ Transfection Reagent** (SABiosciences, Catalog No. SA-01) for ready-to-transfect expression vectors
- **SureENTRY™ Transduction Reagent** (SABiosciences; Catalog No. SA-02) for ready-to-transduce lentiviral expression vectors
- **APX Centry DD-Protein Stabilization Reagent** (SABiosciences; Catalog No. SA-03) for controlling the expression of DD-tagged proteins
- Opti-MEM™ I Reduced-Serum Medium (Invitrogen)

IV. Protocol:

A. Before the Experiment:

Optimization of transfection conditions (For Plasmids): Optimizing transfection conditions for each cell type is important for the success of an experiment. Variables to consider when optimizing the transfection conditions include cell density, cell viability, amount of DNA, ratio of DNA to transfection reagent, transfection complex formation time, and transfection incubation time (see the detailed protocols for SABiosciences' recommendations). The constitutive expressing CMV-mGFP vector (SABiosciences; Cat # CCS-PCG) can be used to determine the optimal transfection conditions.

Optimization of transduction conditions (For Lentiviral Particles): Optimization of transduction conditions for each cell type is important for successful lentivirus particle use. Variables to consider, when optimizing the transduction conditions include Multiplicity of Infection (MOI), concentration of SureENTRY™ Transduction Reagent used, time of assay development and the cell density. The Cignal Lenti positive control (GFP) (SABiosciences; Cat # CLS-PCG) can be used for determining the optimal transduction conditions.

Multiplicity of Infection (MOI) (For Lentiviral Particles): The transduction efficiency of APX expression vectors varies significantly for different cell types. Users should determine the Multiplicity of Infection (MOI), which is the number of transducing lentiviral particles per cell, required for desired transduction efficiency of a new cell type. The MOI is typically adjusted by increasing or decreasing the amount of virus added per well to a series of wells containing the same number of cells. SABiosciences recommends testing the Cignal

Lenti Positive Control (CLS-PCG) at MOIs of 5, 10, and 50 (each MOI in triplicate), in order to establish the optimal MOI for each cell type to be studied.

To calculate:

$$\text{Multiplicity of Infection (MOI)} = \frac{\text{Number of transducing units (TU) deposited in a well}}{\text{Number of target cells present in that well}}$$

Total transducing units needed per well (TU) = (Total number of cells per well) x (Desired MOI)

$$\text{Total mL of lentiviral particles to add to each well} = \frac{\text{Total TU needed per well}}{\text{TU/mL reported on Certificate of Analysis}}$$

SABiosciences has found that some commonly used cell lines (like HT1080, HEK293 and HepG2 etc.) can be effectively transduced using an MOI between 10 and 25, however, some cell types (like primary cells) are more resistant to transduction and efficient transduction of these cell types may require a higher MOI (~ 50).

Suspending Centry (For DD-tagged proteins):

1. Dispense 100 µl of 100% Ethanol into the tube containing dried Centry Reagent.
2. Close the cap on the Centry Reagent tube.
3. Resuspend the Centry Reagent by tapping or flicking the tube several times.

Optimizing Centry concentration (For DD-tagged proteins): Centry is the ligand responsible for stabilizing DD-tagged protein. The final concentration of Centry used in the experiment depends on the type of cells and DD-tagged APX vector used in the experiment. The *in vivo* amount of DD-tagged protein can be adjusted by varying the amount of Centry in a culture media. SABiosciences recommends determining a suitable amount of Centry by initially testing using Centry in a range of 50 nM to 1 µM in a culture media.

Optimizing Centry incubation time (For DD-tagged proteins): The specific level of DD-protein accumulation in cells not only depends upon the concentration of Centry, but also incubation times. SABiosciences recommends performing a time course experiment in order to determine the optimal incubation time with Centry.

Stable cell generation: The lentivirus and plasmid APX vectors have selection markers for generating stable cell lines. DYKDDDDK-tagged ORF expression plasmids and DD-tagged ORF adjustable expression plasmids have Neomycin as a selection marker and lentiviral particles expressing DYKDDDDK-tagged ORF have Puromycin as a selection marker, whereas lentiviral particles expressing DD-tagged ORF have turboGFP as a selection marker for generating stable cell lines.

B. Transfection protocol for DYKDDDDK-tagged ORF expression plasmids

The following protocol is designed to transfect the adherent cell line, HEK-293H, using SureFECT Transfection Reagent (Cat. # SA-01) in a 96-well plate format. Transfection reagent from other vendors can be used with the APX plasmids. *If you are using a transfection reagent other than SureFECT follow the manufacturer's protocol.* Moreover, the use of plates or wells of different size require the user to adjust the components in proportion to the surface area of the new plate or well. **The protocol below is just a general guideline; the optimal conditions/amounts should be adjusted according to the cell type and the study requirements.**

Read the protocol completely before starting the experiment.

1. One day before transfection, seed 1-1.5×10^4 cells in each well of 96-well plate with 100 µl of growth medium.

2. On the day of transfection, add 200 ng of DYKDDDDK expression vector or the negative control expression vector (SABiosciences; Cat. # DA0-000A) into separate 25 µl aliquots of Opti-MEM I Reduced-Serum Medium. Mix gently and incubate mixture for 5 minutes at room temperature.

3. For each well, add 0.3 µl of SureFECT into 25 µl of Opti-MEM separately. Mix gently and incubate mixture for 5 minutes at room temperature.

4. Add 25 µl of SureFECT mix to 25 µl DYKDDDDK expression vector mix. Mix gently and incubate for 20 minutes at room temperature.

5. Add 50 µl "DYKDDDDK expression vector-SureFECT complexes" in medium to the appropriate well containing cells and 100 µl of normal growth medium. This gives a final volume of 150 µl. Mix gently by rocking the plate back and forth.

6. Incubate cells at 37°C in a 5% CO2 incubator for 16-24 hours.

7. After 16-24 hours of transfection, change the medium to complete growth medium and carry out a desired study.

C. Transfection protocol for DD-tagged ORF adjustable expression plasmid

The following protocol is designed to forward transfect adherent cell line, HEK-293H, using SureFECT Transfection Reagent (Cat. # SA-01) in a 6-well plate format. The APX expression vectors works well with transfection reagent from other vendors. *If you are using a transfection reagent other than SureFECT follow the manufacturer's protocol.* Moreover, if you are using plates or wells of different size, adjust the components in proportion to the surface area. **This is just a general guideline; the optimal conditions/amounts should be adjusted according to the cell type and the study requirements.**

Read the protocol completely before starting the experiment.

1. One day before transfection, seed 7.5×10^5 cells in each well of 6-well plate with 2 ml of growth medium.

2. On the day of transfection, add 250 ng - 1 µg* of APX vector or positive control turboGFP expression vector (SABiosciences; Catalog No. DB4-001A) into separate 250 µl aliquots of Opti-MEM. Mix gently and incubate mixture for 5 minutes at room temperature.

*Note: In order to have low amounts of expressed DD-fusion protein in the absence of Centry reagent, SABiosciences recommends either using small amounts of DNA or preferably generating stable cell lines for improved performance.

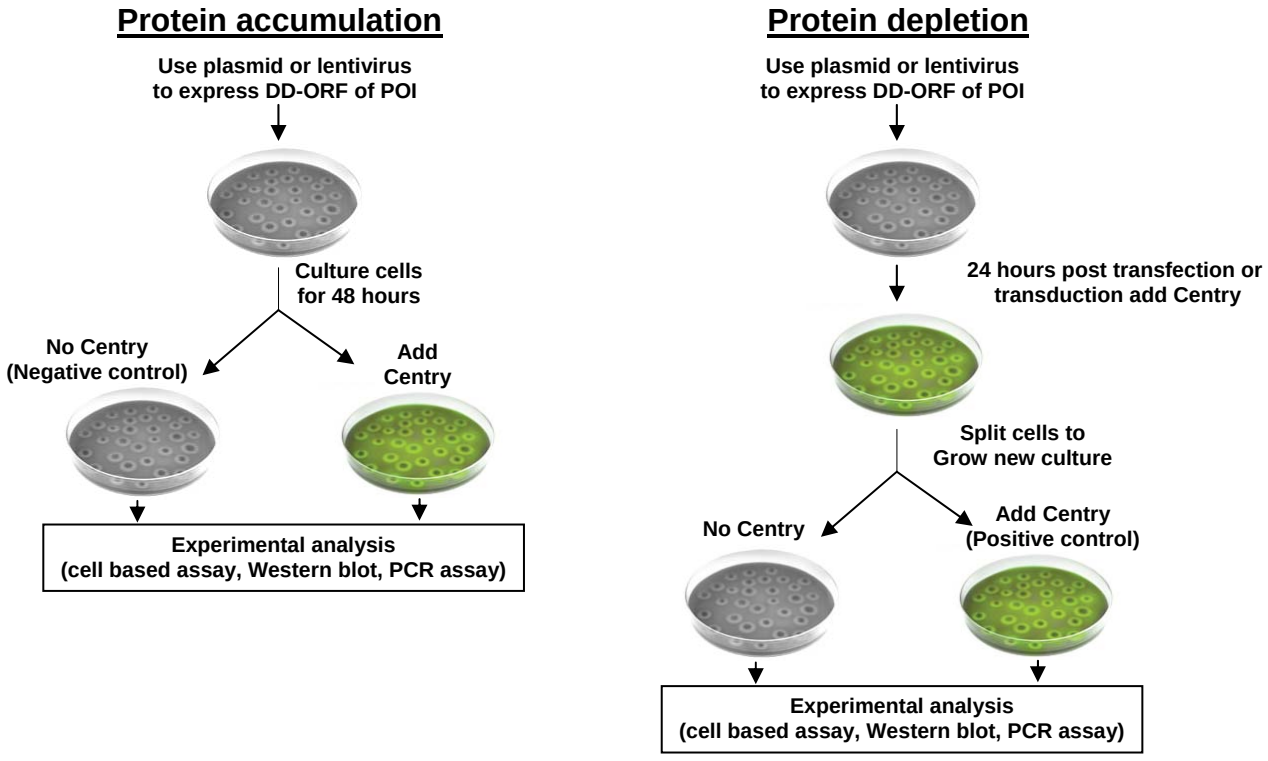
3. For each well, add 7.5 µl of SureFECT into 250 µl of Opti-MEM separately. Mix gently and incubate mixture for 5 minutes at room temperature.

4. Add 250 µl of SureFECT mix to 250 µl APX vector mix. Mix gently and incubate for 20 minutes at room temperature.

5. Add 500 µl "APX vector-SureFECT complexes" in medium to the appropriate well containing cells and 2 ml of normal growth medium. This gives a final volume of 2.5 ml. Mix gently by rocking the plate back and forth.

6. Incubate cells at 37°C in a 5% CO2 incubator for 16-24 hours.

7. After 16-24 hours of transfection, split the cells into at least two parallel cultures in a complete growth medium.



8. **DD-tagged protein accumulation**: Twenty-four hours post cell splitting, treat one culture with the Centry (50 nM to 1 µM) and use another as an untreated negative control (The added Centry reagent will protect your DD-tagged protein from proteasomal degradation, resulting in a rapid accumulation within the cell) and carry out your desired study.

DD-tagged protein depletion: The presence of DD-tag enables rapid accumulation as well as depletion of protein of interest. The Centry molecule can bind to the DD and stabilize the protein. Likewise, removal of Centry reagent can cause rapid destabilization and proteasomal degradation of protein.

9. After incubating transfected cell with Centry reagent for desired time (which results in the accumulation of DD-tagged protein), split the cells into at least two parallel cultures in a complete growth medium.

10. Continue with the presence of Centry in one culture (consistently having high level of protein of interest) whereas culture other plate without Centry and carry out the desired study.

D. Transduction protocol for lentiviral particles expressing DYKDDDDK-tagged ORF

The following protocol is designed to transduce HEK-293 cells using lentiviral particles expressing DYKDDDDK-tagged ORF in a 96-well plate format. If you are using plates or wells of different size, adjust the components in proportion to the surface area. **This is just a general guideline; the optimal transduction conditions should be optimized according to the cell type and the study requirements**.

Read the protocol completely before starting the experiment.

1. One day before transduction, seed ($0.5\text{-}1\times10^4$ cells) in each well of 96-well plate.

2. On the day of transduction, remove medium from wells. To each well add 20 µl of lentiviral particles expressing the protein of interest or negative control lentiviral particles (cat #DN9-000A) adjusting to a final volume of 50 µl using growth medium without antibiotics (DMEM with 10% FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate). In this particular case, add 30 µl of growth medium without antibiotics.

3. Add SureENTRY Transduction Reagent to a final concentration of 8 µg/ml in each well. Gently swirl the plate to mix.

4. Incubate for 18-20 hours at 37°C in a humidified incubator in an atmosphere of 5% CO2.

5. 24 hours post transduction, remove the medium containing lentiviral particles from wells. Add 100 µl of fresh growth medium (DMEM with 10% FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 100 U/ml Penicillin and 100 µg/ml Streptomycin) to each well and carry out the desired study.

Note:

a. SureENTRY Transduction Reagent enhances transduction of most cells, however, some cells like primary neurons are sensitive to the SureENTRY Transduction Reagent. Do not add SureENTRY Transduction Reagent to these types of cells. If working with a cell type for the first time, a SureENTRY Transduction Reagent control only well should be used to determine cell sensitivity.

b. When transducing Cignal lentiviral particles into a cell type for the first time, we suggest using either 10 or 50 MOI as a starting point to determine the optimal assay development

conditions. Always include Cignal Lenti Positive Control (GFP) (SABiosciences; Cat. # CLS-PCG) for determining transduction efficiency.

E. Transduction protocol for lentiviral particles expressing DD-tagged ORF

The following protocol is designed to transduce HEK-293 cells using lentiviral particles expressing DD-tagged ORF in a 96-well plate format. If you are using plates or wells of different size, adjust the components in proportion to the surface area. **This is just a general guideline; the optimal transduction conditions should be optimized according to the cell type and the study requirements**.
Read the protocol completely before starting the experiment.

1. One day before transduction, seed (0.5-1×10^4 cells) in each well of 96-well plate.

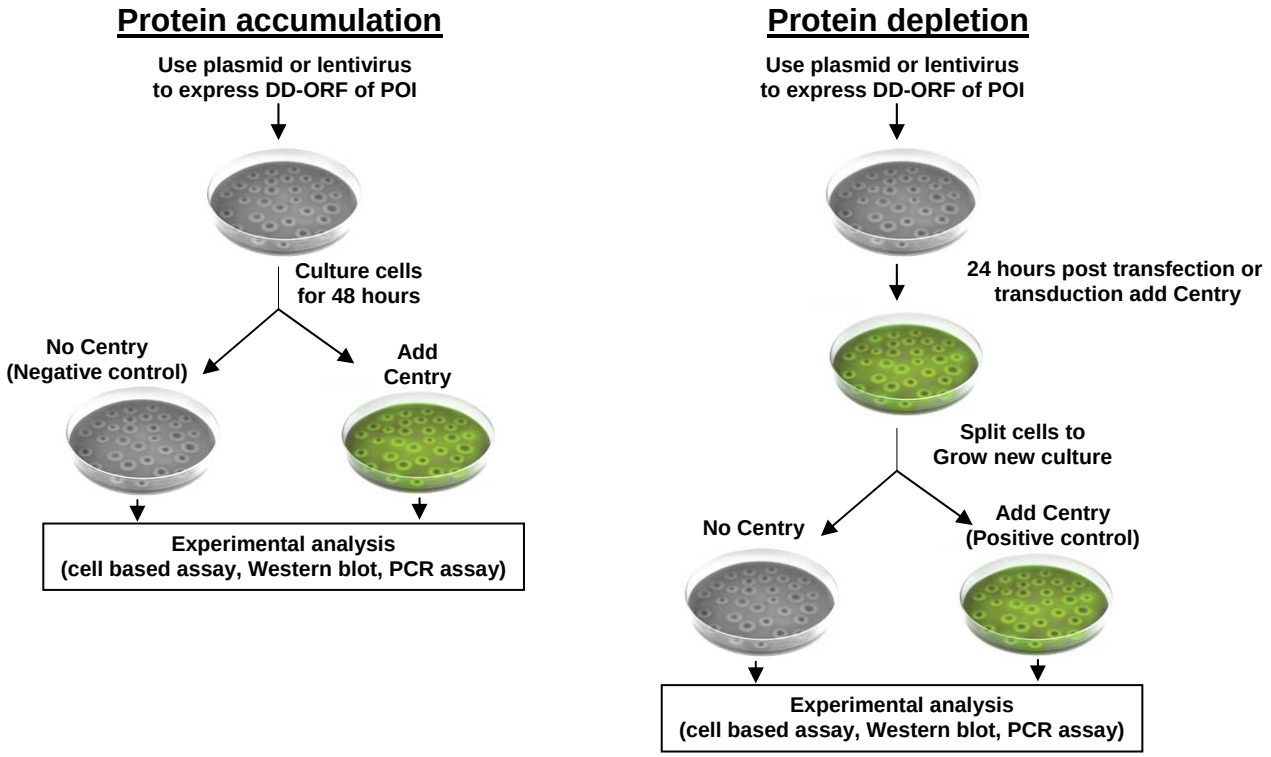
2. On the day of transduction, remove medium from wells. To each well add 10 µl of lentiviral particles expressing protein of interest* or positive control tGFP (cat #DO3-001A) and make up the total volume of 50 µl using growth medium without antibiotics (DMEM with 10% FBS, 0.1mM NEAA, 1mM Sodium pyruvate).

* Note: In order to have low amounts of expressed DD-fusion protein in the absence of Centry reagent, SABiosciences recommends either using small amount of lentivirus or preferably generating stable cell lines for improved performance.

3. Add SureENTRY Transduction Reagent to a final concentration of 8 µg/ml in each well. Gently swirl the plate to mix.

4. Incubate for 18-20 hours at 37°C in a humidified incubator in an atmosphere of 5% CO2.

5. Twenty-four hours post transduction, split the cells into at least two parallel cultures in a complete growth medium.



6. **DD-tagged protein accumulation**: Twenty-four hours post cell splitting, treat one culture with the Centry (50 nM to 1 µM) and use another as an untreated negative control (The added Centry will protect your DD-tagged protein from proteasomal degradation, resulting a rapid increase in its level in cell) and carry out your desired study.

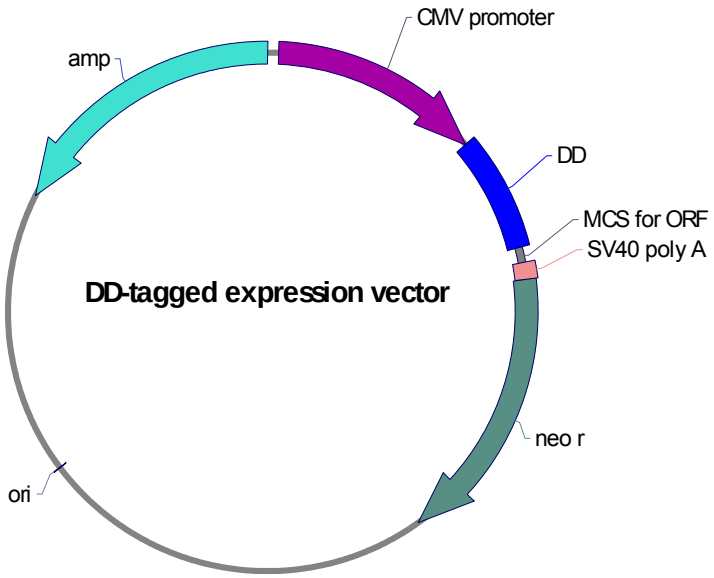
DD-tagged protein depletion: The presence of DD-tag enables rapid accumulation as well as depletion of protein of interest. The Centry molecule can bind to DD to stabilize the protein. Likewise, removal of Centry reagent can cause rapid destabilization and proteasomal degradation of protein.

7. After incubating transfected cell with Centry for desired time (which result in accumulation of protein of interest), split the cells into at least two parallel cultures in a complete growth medium.

8. Continue with the presence of Centry in one culture (consistently having high level of protein of interest) whereas culture other plate without Centry and carry out the desired study.

V. Cloning Protocol:

Cloning Of Open Reading Frame of Your Choice into DD-Tagged Expression Vector



MCS for ORF :

AgeI SgfI AscI RsrII EcoRI MluI PmeI
ACCGGTGCGATCGCCGGCGCGCCCGGACCGTCGAATTCACGCGTTAAGTTTAAAC

T G A I A

There are several rare-cutting restriction enzymes in the Multiple Cloning Site (MCS) of DD-tagged expression vector, such as SgfI, AscI, RsrII, MluI and PmeI. Most of mammalian Open Reading Frame (ORF) lacks sites for these rare-cutting restriction enzymes. We recommend scanning the ORF of your protein of interest for these restriction enzyme sites and select a pair for cloning. ORF must be PCR amplified using primers appending rare cutting restriction enzyme cloning sites, and then cloned into linearized vector.

Here is an example using SgfI and MluI pair for directional cloning.

1. Primer design:

Forward primer with SgfI:
5’ GAGGCGATCGCCNNNNNNNNN…..NN 3’
Ns (20-35 bp) represent the sequence of ORF of protein of interest beginning with the start codon, ATG. SgfI site is underlined and an additional “C” base after it is designed to keep reading frame with N-terminal DD tag.

Reverse primer with MluI:
5’ GCGACGCGTNNNNNNNNn…..NN 3’
Ns (20-35 bp) represent the reverse complement sequence of ORF of protein of interest beginning with the stop codon.

If SgfI or MluI are present in the Open Reading Frame, other restriction enzymes, like AscI, RsrII and PmeI, can be used. Since DD is N-terminal tag, in the Forward primer, additional base(s) after restriction enzyme site may be necessary to maintain reading frame. Arbitrary bases (3-4) should be added at the 5’ of both primers to ensure efficient restriction enzyme cutting.

2. PCR amplification of ORF:

To minimize the mutations during the PCR amplification, a high fidelity DNA polymerase, such as *Pfu* DNA polymerase, is recommended. Follow manufacture’s protocols to generate PCR product. Confirm the size of the PCR product by agarose gel electrophoresis and purify the reaction using a purification column (such as PCR purification kit). Elute the purified product in 30μl elution buffer (10mM Tris.Cl, pH 8.5).

3. Cloning of ORF into the empty vector:

Digestion of purified PCR product with SgfI and MluI:

Purified PCR product	28 μl
10× NEB restriction buffer 3	4 μl
100× BSA	0.4 μl
SgfI (80U/μl)	0.2 μl
MluI (10U/ μl)	0.8 μl
H_2O	make up to 40 μl

Incubate at 37°C for 3 hours. Then purify the digested PCR product using a DNA purification column (such as PCR purification kit) and elute in 30 μl elution buffer (10mM Tris.Cl, pH 8.5) and measure the concentration.

Digestion of Empty Vector with SgfI and MluI:

Vector DNA (0.5 μg/ μl)	2 μl
10× NEB restriction buffer 3	20 μl
100× BSA	2 μl
SgfI (80U/ μl)	0.75 μl
MluI (10U/ μl)	4 μl
H_2O	make up to 200 μl

Incubate at 37°C for 3 hours and then at 80°C for 20 minutes.

Dephosphorylation of empty vector:

Add 1/10 volume of 10× antarctic Phophatase Reaction Buffer and 1 μl of Antarctic phosphatase (NEB) and continue incubation at 37°C for 1 hour. Heat inactivate for 5

minutes at 65°C, following by purifying on a column and eluting in 30 µl of elution buffer (10 mM Tris.Cl, pH 8.5).

Set up ligation reaction with purified vector and PCR product:

Vector (50 ng)	- µl
Insert (PCR product)	- µl *
2× Quick ligation reaction buffer (NEB)	10 µl
H_2O	make up to 20 µl

Mix and add 1 µl Quick T4 DNA ligase (NEB)** and incubate at room temperature for 5 minutes and proceed with transformation.

* based on the size of PCR product, try to maintain the molar ratio from 3:1 to 6:1 (insert: vector)
** Other DNA ligases can be substituted.

Transformation

Transform high-efficiency *E.coli* competent cells (≥ 10^8 cfu/ µg DNA) with 2 µl of the ligation reaction according to manufacture's protocol. Plate the transformants on LB-agar plates supplemented with 100 µg/ml ampicillin.

Pick up 4 colonies for doing miniprep. Confirm the correct colony by restriction enzyme digestion and sequencing.

5' CMVDD sequencing primer: (5' AAA CTG ACT ATA TCT CCA GA 3')

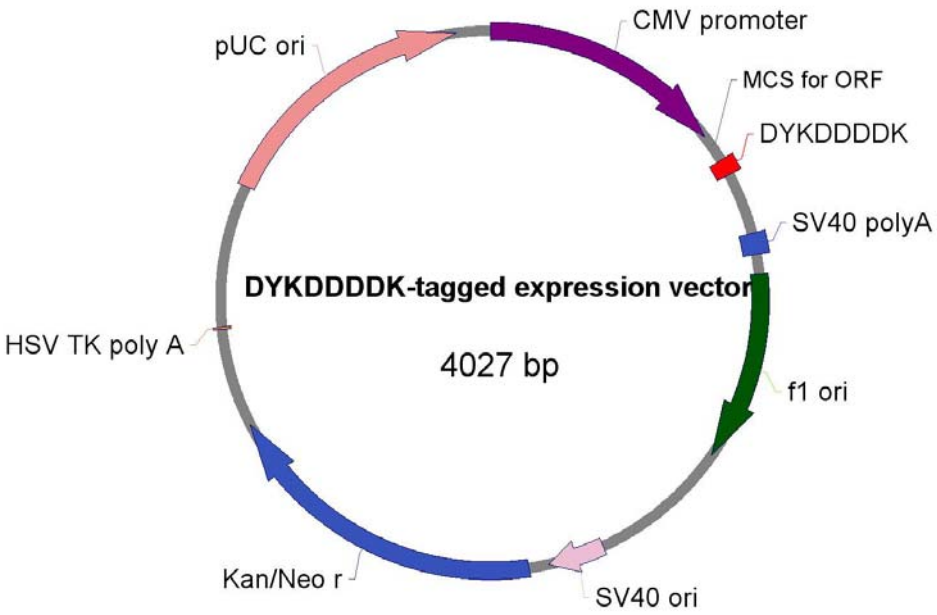
3' CMVDD rev sequencing primer: (5' TTC TAG TTG TGG TTT GTC CA 3')

VI. Troubleshooting and FAQs

If you have questions, please check our website (www.SABiosciences.com) for a more complete listing of Frequently Asked Questions (FAQs), or call our Technical Support Representatives at 1-888-503-3187 or 301-682-9200.

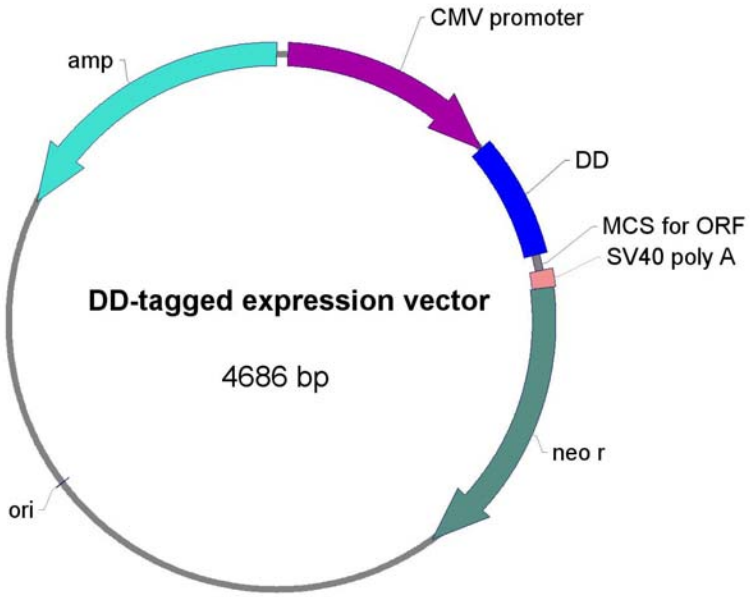
Appendix: Vector Maps

1. Vector map of DYKDDDDK tagged ORF expression plasmid:



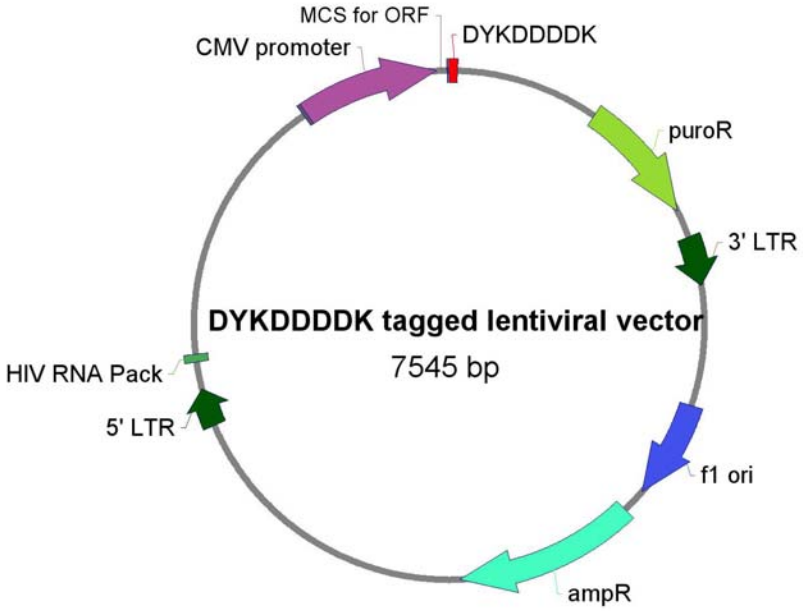
MCS: Multiple cloning site; ORF: open reading frame

2. Vector map of DD-tagged ORF expression plasmid:



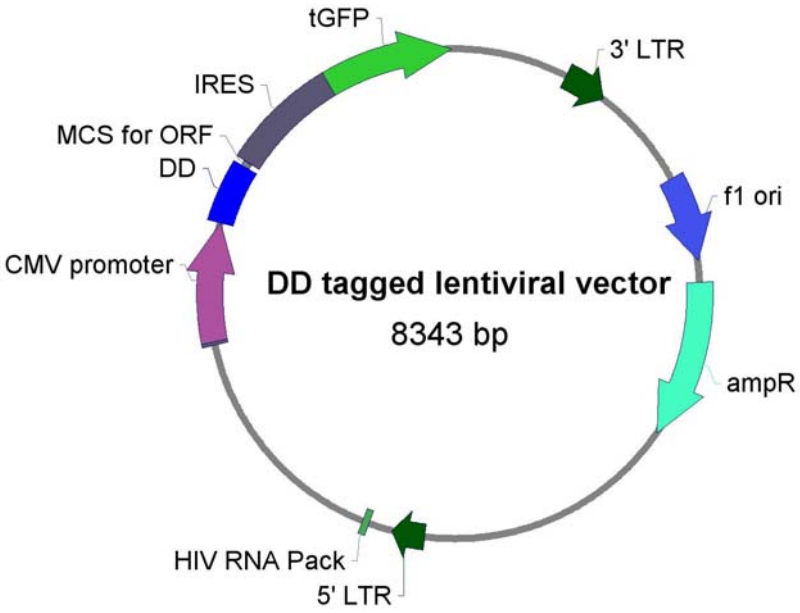
MCS: Multiple cloning site; ORF: open reading frame

3. Vector map of lentiviral plasmid expressing DYKDDDDK-tagged ORF:



MCS: Multiple cloning site; ORF: open reading frame

4. Vector map of lentiviral plasmid expressing DD-tagged ORF:



MCS: Multiple cloning site; ORF: open reading frame

Notes:

APX System User Manual

Part #1050A Version 1.1 10/2/2009

BIOMOL GmbH
Waidmannstr. 35
22769 Hamburg
info@biomol.de
www.biomol.de
Phone: +49-40-8532600 or 0800-2466651 (D)
Fax: +49-40-85326022 or 0800-2466652 (D)